[Frontiers in Bioscience 7, d970-977, April 1, 2002]

RYANODINE RECEPTORS, FKBP12, AND HEART FAILURE

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1. ABSTRACT

RyR2 function is regulated by highly conserved signaling pathways that modulate excitation-contraction (EC) coupling. cAMP dependent protein kinase (PKA) phosphorylation of RyR2 plays an important role in regulating channel function in response to stress signaled by the sympathetic nervous system (the classic "fight or flight response") (1). PKA phosphorylation of RyR2 induces dissociation of the regulatory protein FKBP12.6 resulting in channels with increased sensitivity to Ca^{2+}-induced Ca^{2+} release. Under normal physiological conditions (no cardiac damage) PKA phosphorylation of RyR2 is part of an integrated physiological response that leads to increased EC coupling gain and increased cardiac output. PKA-hyperphosphorylation of RyR2 in failing hearts is a maladaptive response that results in depletion of FKBP12.6 from the RyR2 macromolecular complex and defective channel function (pathologically increased sensitivity to Ca^{2+}-induced Ca^{2+} release) that may cause depletion of SR Ca^{2+} and diastolic release of SR Ca^{2+} that can initiate delayed after depolarizations (DADs) that trigger ventricular arrhythmias (1). RyR2 mutations in patients with catecholaminergic induced sudden cardiac death provide further evidence linking the sympathetic nervous system, RyR2 and ventricular arrhythmias (2-4). The chronic hyperadrenergic state of heart failure is associated with defective Ca^{2+} signaling in part due to PKA hyperphosphorylation of RyR2.

2. INTRODUCTION

Although we now understand a great deal more about the systems that regulate cardiac function than we did 20 years ago, this abundance of new knowledge brings with it additional challenges. It is becoming ever more apparent that the concept of linear signaling pathways does not apply to complex biological systems. In this light disease states that perturb normal signaling often cannot be explained by a single defect in one pathway. Accordingly, therapies of many diseases likely will not succeed when they are targeted only at one pathway in a complex parallel signaling system. This review focuses on the ryanodine receptor (RyR2)/ calcium (Ca^{2+}) release channel, on the cardiac sarcoplasmic reticulum (SR). The RyR2 is one component of a complex integrated system that regulates cytosolic [Ca^{2+}] which in turn controls cardiac muscle contraction and relaxation. Many other molecules exert regulatory effects on Ca^{2+} homeostasis mechanisms in cardiomyocytes. It is beyond the scope of this focused review to address the full complexities of this regulatory system and interested readers are encouraged to read further on the subject of cardiac muscle function to get an overview that will help place the present discussion in the proper global context.

3. RYANODINE RECEPTORS: STRUCTURE AND FUNCTION

RyRs are members of a gene family that includes three isoforms: RyR1, expressed predominantly in skeletal muscle but also in non-muscle including the central nervous system; RyR2, expressed predominantly in cardiac muscle but also in non-muscle including the central nervous system; and RyR3, expressed in specialized muscle and non-muscle tissues including the brain. Primary structures of the three RyR isoforms have been deduced from their corresponding cDNAs (5-8) revealing >65% amino acid sequence homology among them. Homotetramers of four RyRs, each with a molecular mass of ~560,000 Da, comprise a single RyR channel with a molecular mass in excess of 2.3 million

Da. RyR channels have enormous cytoplasmic domains containing binding sites for proteins and other channel modulators (e.g. Ca^{2+}) that control the state of activity of the channel-forming domain of the molecule.

Intact RyR have been isolated and purified from skeletal (9-11) and cardiac muscles (12,13) after mild CHAPS solubilization of heavy SR using ^3H ryanodine as a high affinity ligand. Recombinant RyR1 has been expressed in insect cells (14), *Xenopus oocytes* (15), HEK cells (16,17), and CHO cells (18). Purified receptor has been incorporated in planar lipid bilayers and demonstrates characteristics identical to the Ca^{2+} release channel of the heavy SR (9,17,19-23). The activity of RyR is modulated by a variety of agents. Cytoplasmic Ca^{2+} activates the channel at nM-μM concentrations, while mM concentrations inhibit the channel (24). Channels are activated by ATP (25) and inhibited by Mg^{2+} (26). In addition to these physiological regulators, useful modulators of RyR channel function include caffeine, specific antibodies (27,28), ryanodine (29-31), ruthenium red, and the immunosuppressant drugs FK506 and rapamycin (14,32-34).

The regulation of cardiac EC coupling by the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR2) in cardiomyocytes, known as Ca^{2+}-induced Ca^{2+} release (CICR), has been appreciated for more than a decade (35,36) and it is well known that the amplitude of the Ca^{2+} transient generated by SR Ca^{2+} release determines contractile force in cardiomyocytes. Systems that regulate SR Ca^{2+} release include: 1) the triggers (predominantly Ca^{2+} influx through the voltage-gated Ca^{2+} channel on the plasma membrane and secondarily Ca^{2+} influx via the Na^+-Ca^{2+} exchanger); 2) the SR Ca^{2+} release channel or type 2 ryanodine receptor (RyR2); 3) the SR Ca^{2+} reuptake pump (SERCA2a) and its regulator phospholamban. These trigger, release, reuptake systems are modulated by signaling pathways including the beta adrenergic receptor (β-AR) signaling pathway (i.e. phosphorylation by PKA).

3.1. RyR2 complex is formed via leucine/isoleucine zippers

RyR1 and RyR2 contain highly conserved leucine/isoleucine zippers (LIZs, α helical heptad repeats of Leu/Ile residues) that bind to LIZs present in the adaptor/targeting proteins for kinases (PKA) and phosphatases (PP1 and PP2A) that regulate RyR channel function and play a role in defects in channel function in heart failure (1,37). A heptad repeat of hydrophobic residues is the important feature of LIZs (38). Leu/Ile line up on one face of the helix (3.5 residues/turn) and oligomerize with other coiled coil helices. A 2nd important feature of LIZs is that hydrophobic residues occupy the "*a*" as well as the "*d*" (Leu/Ile) positions of the repeating heptad of *a-g* residues that form the helix. Leu residues can be replaced by Ile or Val and can include "skips" and "hiccups" (39). Specificity is imparted via the amino acids in the other positions in the coiled-coil forming α helix. We have identified leucine heptad repeats in the cardiac RyR2 and the skeletal RyR1 that are involved in targeting kinases and phosphatases to the channel (37) and the targeting of PKA, PP2A and PP1 to RyR2 can be disrupted by mutating these leucine zipper motifs (37).

Elucidating the role of LIZs in mediating the targeting of specific kinases and phosphatases to RyR2 provides tools that can be used to specifically eliminate PKA-dependent modulation of RyR2 function. We have used a specific RyR2 peptide containing LIZ3 to compete the mAKAP/PKA complex from the channel (37) demonstrating that it is possible to specifically determine the effects of PKA phosphorylation of RyR2 (37). This approach can be adapted to *in vivo* studies by expressing the specific peptides in cardiomyocytes to determine the effects of inhibiting PKA phosphorylation of RyR2 in intact cardiomyocytes (37).

3.2. FKBP12.6 regulates RyR2

A 12 and a 12.6-kDa protein respectively are tightly associated with highly purified RyR1 and RyR2 and modulate the function of the channels, possibly by enhancing cooperativity among its four subunits (5,14,33,40-42). The 12 kDa protein, was originally identified as a peptide KC7 that co-purifies with RyR1 (5), and was later shown to be FKBP12, the cytosolic binding protein for the immunosuppressant drugs FK506 and rapamycin (43). FKBP12 is expressed at high levels in all muscles (40). FKBP12 and FKBP12.6 are *cis-trans* peptidyl-prolyl isomerases and are members of the immunophilin family of proteins (42). The molar ratio of FKBP12 to RyR1 in highly purified RyR1 preparations is ~1:1 (44), indicating that one FKBP12 molecule binds to each subunit of the Ca^{2+}-release channel or four FKBP12 molecules are part of each RyR1-channel complex.

In the absence of FKBP12/12.6, RyR1/RyR2 channels exhibit partial openings known as subconductance states. However, when RyR1 and FKBP12 were co-expressed in insect cells, these subconductance states are eliminated (14). Similarly when FKBP12.6 was removed from RyR2 with FK506 or rapamycin subconductances appeared (33). These results indicate a cellular function for FKBP12/12.6 and establish that functional Ca^{2+}-release channels are a complex composed of RyR and FKBP. These results have recently been confirmed by other laboratories (45-47). In addition to stabilizing RyR channels, FKBPs are required for coupled gating (48,49); a phenomenon in which two or more physically connected RyR channels can gate simultaneously. Coupled gating provides a mechanism for the coordinated activation and inactivation of RyR channels (and thus Ca release) during EC coupling (48,49).

4. PKA PHOSPHORYLATION REGULATES RYR2 FUNCTION

Activation of the sympathetic nervous system in response to stress raises circulating catecholamines that activate β-adrenergic receptors and elevate intracellular cAMP. Among the many targets for the cAMP-dependent kinase PKA are the trigger for EC coupling, the L-type channel, the release channel, RyR2, and phospholamban which regulates the Ca^{2+}-uptake pump SERCA2a (Figure1A).

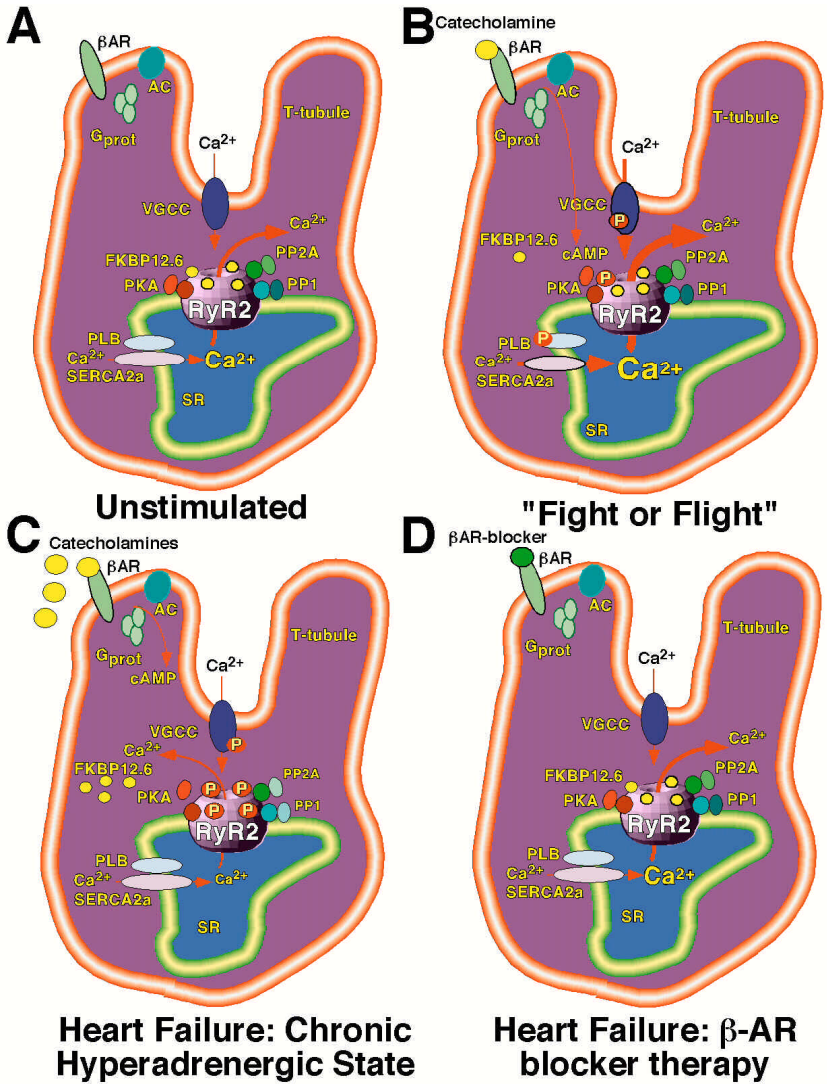

Figure 1. Modulation of cardiac excitation-contraction coupling by PKA phosphorylation. **A)** Key features of cardiac EC coupling are show. Action potentials depolarize the transverse tubule (T-tubule) and activate voltage-gated Ca^{2+} channels (VGCC). Ca^{2+} influxes through VGCC and activates RyR2 (SR Ca^{2+} release channel). RyR2 releases Ca^{2+} from the sarcoplasmic reticulum (SR) raising $[Ca^{2+}]_{cyt}$ from ~100 nM to ~1 μM. Ca^{2+} binding to troponin C induces a conformational change that activates muscle contraction. Ca^{2+} is pumped back into the SR by the Ca^{2+}-ATPase (SERCA2a). SERCA2a is inhibited by phospholamban (PLB). RyR2 macromolecular complexes include four RyR2, each RyR2 binds one FKBP12.6, PKA catalytic and regulatory subunits (RII) and mAKAP, PP2A and its targeting protein PR130, PP1 and its targeting protein spinophilin (only one of the four of each of these proteins are shown except in 1C where four FKBP12.6 are shown) (1,37). β-adrenergic receptor (β-AR) signaling pathway is shown including β-AR in the plasmamembrane that activates adenylyl cyclase (AC) via G proteins (G_{prot}). **B)** The "fight or flight" response involves activation of the sympathetic nervous system resulting in the release of catecholamines into the circulation that activate β-AR and elevate cAMP levels that in turn activate PKA (by causing the release of the PKA catalytic subunit from its regulatory subunit) (1). PKA phosphorylates and activates: 1) the VGCC, increasing Ca^{2+} influx which activates RyR2; 2) RyR2, increasing Ca^{2+}-dependent activation thereby increasing EC coupling gain; 3) PLB, releasing inhibition of SERCA2a and increasing SR Ca^{2+}-uptake. Increasing EC coupling gain increases cardiac output. C) Decreased cardiac function in heart failrue results in chronic activation of the fight or flight response (sympathetic nervous system) because the damaged heart cannot adequately respond to increase cardiac output. The chronic hyperadrenergic state results leading to PKA hyperphosphorylation of RyR2 (1). PKA hyperphosphorylation is associated with reduced PP1 and PP2A levels in the RyR2 complex and depletion of FKBP12.6 from the RyR2 complex. This pathologically increases Ca^{2+}-dependent activation of RyR2 resulting in depletion of SR Ca^{2+} stores, uncoupling of RyR2 from each other (further reducing EC coupling gain) and potentially providing diastolic SR Ca^{2+} release that can activate depolarizations that trigger fatal ventricular cardiac arrhythmias. D) β-AR blockade restores FKBP12.6, PP1 and PP2A levels and RyR2 function in failing hearts (61).

Thus, activation of the sympathetic nervous system ("fight of flight" response) increases cardiac output by increasing the gain of EC coupling due to PKA phosphorylation that activates the trigger, the release channel and the SR Ca^{2+}-uptake pathway. PKA phosphorylation of RyR2 is physiologically regulated *in vivo* in response to activation of the sympathetic nervous system ("fight or flight" response) and channels from failing hearts are PKA hyperphosphorylated (due to a maladaptive response that creates a chronic hyperadrenergic state) resulting in dissociation of the FKBP12.6 and altered channel function (1). Maladaptation of this physiological pathway, that has never been subjected to evolutionary pressure since heart failure is a new disease of the last century or two, plays an important role in the pathophysiology of heart failure (50,51).

Stimulation of the sympathetic nervous system results in phosphorylation of RyR2 by PKA and activation of the channel (Figure 1B). PKA phosphorylation potently modulates RyR2 function and is physiologically regulated *in vivo* (1,37,50-59). PKA hyperphosphorylation of RyR2 in failing hearts shifts the sensitivity of RyR2 to Ca^{2+}-induced Ca^{2+} release to the left (1) resulting in "leaky" channels (Figure 1C) (channels with increased sensitivity to Ca^{2+}-induced Ca^{2+} release) that may cause diastolic Ca^{2+} release that generates delayed after depolarizations that trigger VT (50,51). SR Ca^{2+} leak during diastole can generate "delayed after depolarizations" that can trigger fatal cardiac arrhythmias (e.g. VT).

4.1. PKA Phosphorylation of RyR2 dissociates FKBP12.6 from the channel

The RyR2 macromolecular complex includes FKBP12.6, PKA and its targeting protein mAKAP, PP1 and its targeting protein spinophilin and PP2A with its targeting protein PR130 (1,37). Binding of FKBP12.6 to RyR2 is physiologically regulated by PKA phosphorylation of the channel which dissociates FKBP12.6 from RyR2 (1) resulting in increased activity (increased Po) of RyR2. Similarly, PKA phosphorylation of RyR1 also regulates FKBP12 binding (Marks et al in preparation). Increased RyR2 activity induced by dissociation of FKBP12.6 results from a shift to the left in the Ca^{2+} dependence for RyR2 activation. Regulation of FKBP12.6 binding to RyR2 is an important physiological modulator of EC coupling because FKBP12.6 binding to RyR2 is involved in the regulation of EC coupling gain (1,50,51), defined as the amount of SR Ca^{2+} released for a given trigger (Ca^{2+} influx via the voltage-gated Ca^{2+} channel) (60). In failing hearts PKA hyperphosphorylation of RyR2 also decreases coupled gating between RyR2 (48,49) due to dissociation of FKBP12.6 from the channel (unpublished data). Reduced coupled gating can further reduce EC coupling gain and may lead to incomplete closure of RyR2 during diastole (50,51). Administration of oral β-adrenergic receptor blocker (metoprolol 25 mg b.i.d.) reverses the PKA hyperphosphorylation of RyR2, restores normal stoichiometry of the RyR2 macromolecular complex and restores normal single channel function in a canine model of heart failure (61).

RyR2 is PKA phosphorylated on Ser^{2809} (1) and by Ca^{2+}-CaM kinase (CamKII) on the same residue (53). PKA phosphorylation of RyR2 increases the activity (Po) of the channel (1), increases ^{3}H ryanodine binding (a surrogate for RyR2 function) in cardiac myocytes (59) and increases Po of RyR2 channels activated by flash photolysis of caged Ca^{2+} (58). In the latter experiments (58) the steady state Po of PKA treated channels (PKA phosphorylation of RyR2 was not determined in these experiments) was decreased possibly due to subsequent dephosphorylation of RyR2 by bound phosphatases that were not inhibited. PKA dependent phosphorylation reverses Mg^{2+} dependent inhibition of RyR2, and induces subconductance states in planar lipid bilayer experiments (52). Isoproterenol treatment of isolated rat myocytes increases the phosphorylation of RyR2 (59) and the amplitude of Ca^{2+} sparks (62). Other kinases including CamKII phosphorylate RyR2 although there is disagreement as to whether CamKII inhibits or activates RyR2 (52,55,57,63).

Another effect of PKA hyperphosphorylation of RyR2 in failing hearts would be to functionally uncouple the channels from one another. RyR2 are arranged on the sarcoplasmic reticulum membrane in closely packed arrays such that their large cytoplasmic domains contact one another. We have shown that multiple RyR2s can be isolated under conditions such that they remain physically coupled to one another (48,49). When these coupled channels are examined in planar lipid bilayers multiple channels exhibit simultaneous gating termed "coupled gating" (48,49). Removal of the regulatory subunit, FKBP12.6, functionally but not physically uncouples multiple RyR2 channels (49). Coupled gating between RyR2 channels may be an important regulatory mechanism in EC coupling as well as other signaling pathways involving intracellular Ca^{2+} release. This may have important implications for understanding the molecular pathophysiology of heart failure in which PKA hyperphosphorylation of RyR2 which dissociates FKBP12.6 will inhibit coupled gating thereby reducing EC coupling gain and promoting diastolic SR Ca^{2+} leaks (DADs) that can trigger fatal cardiac arrhythmias.

4.2. Cellular affects of PKA phosphorylation of RyR2

There have also been apparently contradictory findings showing that acute treatment with caffeine, which activates RyR2, fails to alter cardiac EC coupling (64), or acute administration of isoproterenol decreases Ca^{2+} spark heterogeneity (65) or improves EC coupling in failing cardiomyocytes (66). Despite the apparent contradictions these findings from other groups are not at odds with our data showing PKA hyperphosphorylation, FKBP12.6 depletion and increased Ca^{2+} sensitivity of RyR2 in failing cardiomyocytes (1). Since heart failure is a chronic disease alterations in RyR2 structure and function in failing hearts persist for months or years (1,50). The consequences of such chronic structural remodeling on RyR2 function are quite distinct from the effects of acute administration of stimulatory compounds such as caffeine or isoproterenol. The acute administration of drugs can transiently modulate RyR2 function allowing other Ca^{2+} handling molecules in the cell to restore homeostasis when RyR2 function returns

to normal. In contrast, the chronic alteration of RyR2 structure and function that occurs in failing hearts can contribute to resetting SR Ca^{2+} content at a lower level due in part to increased "leak" through PKA hyperphosphorylated RyR2. This reduction in SR Ca^{2+} content can contribute to reduced EC coupling gain (1,50). Other alterations that occur in failing hearts such as a decrease in SERCA2a expression and function and/or an increase in Na^{+}/Ca^{2+} exchanger compound these changes as well (e.g. by reducing the amount of Ca^{2+} reuptake into the SR).

It is important to emphasize the distinction between acute administration of isoproterenol versus the chronic hyperadrenergic state of heart failure. We have shown that in heart failure there is an alteration in the stoichiometry of the RyR2 macromolecular complex such that there is a reduction in the amount of phosphatases (PP1 and PP2A) and FKBP12.6 in the complex (1). Moreover, the altered stoichiometry of the RyR2 macromolecular complex is associated with PKA hyperphosphorylation of RyR2 [an increase in the stoichiometry of PKA phosphorylation of the channel from ~1 (normal) to ~3.5 (heart failure) moles of phosphate/mole RyR2] (1). It is unlikely that acute administration of isoproterenol would have the same affects on Ca^{2+} handling as chronic exposure to the hyperadrenergic state of heart failure. For example there might be PKA hyperphosphorylation of RyR2 (but possibly only if phosphatase inhibitors are included), some dissociation of FKBP12.6 from the channel but probably not the decrease in phosphatases in the RyR2 macromolecular complex.

Recently, Litwin and colleagues reported that isoproterenol (100 nmol/L) induced a slight decrease in EC coupling gain and decreased the heterogeneity in Ca^{2+} transients observed in a rabbit infarct model (65). The decrease in EC coupling gain in the infarcted heart is consistent with our data showing PKA hyperphosphorylation of RyR2, which we predict would lead to a reduction in SR Ca^{2+} content (which Litwin and colleagues did not document but which has been shown by others groups in heart failure (67), as opposed to infarct models), as well as uncoupling of coupled RyR2 channels (49).

Critically important in these types of studies is that during isolation of the cardiomyocytes there can be a restoration of normal function due to the ongoing activity of phosphatases in the heart in the absence of phosphatase inhibitors. Our data showing direct targeting of both PP1 and PP2A to RyR2 (1,37) indicate that the phosphatases could be active during cardiomyocyte isolation and could dephosphorylate the channel once the cells are removed from the hyperadrenergic heart failure state in vivo. If this were the case it would explain at least in part why the cells are responsive to isoproterenol and why there is only a modest decrease in EC coupling gain and no decrease in SR Ca^{2+} content. In short there may be a partial restoration of normal function in cardiomyocytes once they are removed from the heart failure milieu in the animal. Moreover, PKA phosphorylation of specific targets within the cardiomyocyte is compartmentalized such that some proteins (e.g. RyR2) are PKA hyperphosphorylated in failing hearts whereas other Ca^{2+} handling proteins such as phospholamban are hypophosphorylated in the same hearts (Marks et al unpublished observation). Our recent data showing that PKA, PP1 and PP2A are specifically targeted to RyR2 via targeting proteins that bind to highly conserved leucine/isoleucine zippers (1,37) on the channel provide strong support for the concept of compartmentalization of PKA signaling.

5. RYR2 MUTATIONS LINKED TO SUDDEN CARDIAC DEATH

Recently eleven RyR2 missense mutations have been linked to two inherited forms of sudden cardiac death (SCD): 1) Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) (2) or Familial Polymorphic VT (FPVT) (3); and 2) Arrhythmogenic Right Ventricular Dysplasia Type 2 (ARVD2) (4). Interestingly, all eleven RyR2 mutations cluster into 3 regions of the channel that correspond to three Malignant Hyperthermia (MH)/Central Core Disease (CCD) mutation regions of the highly homologous skeletal muscle ryanodine receptor/Ca^{2+} release channel RyR1. Both RyR2 mutations linked to genetic forms of catecholaminergic-induced VT and PKA hyperphosphorylation of RyR2 in heart failure may alter the regulation of the channel resulting in increased SR Ca^{2+} leak that triggers SCD. RyR2 mutations linked to exercise-induced SCD may share common functional properties with RyR1 mutations linked to MH and CCD.

The fact that RyR2 mutations have been discovered in individuals with exercise-induced VT suggest an association between PKA phosphorylation of the channel (which is increased by activation of the sympathetic nervous system) and the defective channel function that predisposes to VT. Another intriguing possibility is that PKA phosphorylation of RyR2 exacerbates the defect in mutant RyR2 that are linked to exercise-induced VT.

6. MOLECULAR MECHANISMS UNDERLYING b-ADRENERGIC RECEPTOR BLOCKADE THERAPY FOR HEART FAILURE

β-AR blockade is one of the most effective treatments for heart failure. However, the use of β-AR blockers in patients with heart failure is counterintuitive, as they are known to decrease contractility in normal hearts. We have recently shown that systemic oral administration of a β-AR blocker reverses PKA hyperphosphorylation of RyR2, restores the stoichiometry of the RyR2 macromolecular complex and normalizes single channel function (Figure 1D) in a canine model of heart failure (61). These results may, in part, explain the improved cardiac function observed in heart failure patients treated with β-AR blockers.

7. CONCLUSIONS AND PERSPECTIVES

Elucidation of the molecular basis of cardiac EC coupling has improved our understanding of basic

mechanisms that regulate cardiac function. Applications of these new understandings to the problems of heart failure and cardiac arrhythmogenesis have provided additional new understandings with important therapeutic implications. Complicating these new understandings are the challenging problems of studying integrative physiology using reductionist models. Model systems, that are necessitated by the complexity of cellular and organ physiology, each provide additional challenges that must be overcome. As investigators our goal is to integrate data from all useful models and adapt hypotheses to fit new understandings. To deny the validity of new observations because at first blush they challenge closely held beliefs is an approach that likely leads one into darkness. Most often tomorrow's investigations uncover new threads of understanding that tie together apparently disparate observations. Such an inclusive approach to investigation most often provides pathways towards the light and to lasting truths.

A better understanding of the potential role of PKA hyperphosphorylation of RyR2 in heart failure and its role in the generation of fatal cardiac arrhythmias may emerge from studying the biophysical properties of RyR2 mutations linked to catecholamine-induced ventricular arrhythmias. However, integrating single channel data, cellular and animal physiology and emerging with a unifying mechanism that causes heart failure and SCD will be a challenge. Nevertheless, elucidating the molecular pathogenesis of heart failure and VT will be the basis for strategies that lead to novel therapeutics.

8. ACKNOWLEDGMENTS

The work described in this review was supported by grants from the NIH and the AHA. The author is a Doris Duke Foundation Distinguished Clinical Scientist.

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Key words: Calcium, Excitation-Contraction Coupling, Heart Failure, Review

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